AMENDMENTS TO THE SPECIFICATION

Please replace the paragraph beginning on page 41, line 15 with the amended paragraph that follows below.

Primers 5'EF-2 and mEF-2 were first used to detect the mutations in the Ura+ transformants. Fig. [[6]]5a shows that 9 (clones 1, 2, 3, 12, 13, 14, 33, 40 and 41) of the 12 selected Ura+ clones of JC308 are mEF-2 primer positive, they had a PCR product of the expected size (about 2.2kbp), whereas clones 25, 38 and 47 were negative. As shown in Fig [[6]]5b, when the same clones were analyzed for the presence of wild type sequence with primers EF-2 (1318) and wEF-2, all three mEF-2- clones were wEF-2 primer positive. A 0.8kbp PCR fragment was produced. All mEF-2 + clones were wEF-2- except for clones 33 and 41 that were also wEF-2+. Finally when primers EF2 (1318) and 3'EF-2C were used, all of the selected clones yielded a PCR product of about 1.2 kbp as expected (Fig. [[6]]5c). The PCR products from the clones that were mEF-2+ and wEF-2- were completely digested by Sac II, whereas those of the clones 25, 38 and 47 that were [[mEF-2. but]]mEF-2- and wEF-2+ were not cut by the enzyme. In agreement with being both mEF-2+ and wEF-2+, clones 33 and 41 produced both Sac II clearable and non-clearable PCR produces. To investigate why clones 33 and 41 had both mutated and wild type EF-2, these clones were streaked on new selection plates and let the cells grow to form colonies. Ten well-isolated colonies were picked from each and performed the PCR with primer EF-2 (1318) plus primer 3'EF-2C and the Sac II digestion steps. None of the colonies had the same mixed PCR products as the originals. PCR products of 4 colonies from clone 33, 7 from 41 were completely digested by Sac II, whereas those of other colonies from clones 33 and 41 were not cut at all. This experiment shows that clones 33 and 44 were each originally formed by two different cells, one had an intact EF-2 with the mutations, and the other had an intact wild type EF-2. This experiment was then repeated and checked some of the clones that had only the Sac II clearable EF-2 (clones 1, 2, 3, 12, 13, 14, and 40) and confirmed that they only contained the mutated intact EP-2. After the success in obtaining EF-2 mutant clones of JC308, the same selection procedure was used to identify EF-2 mutant clones of JC303 and JC307. Among the Ura+ positive clones picked for analysis, 35% of

them contained only the mutated intact EF-2. This high frequency of complete mutation may be due to the fact that *Pichia pastoris* only has one copy of EF-2 per haploid genome. As shown for CHO cells and *S. cerevisiae,* the Arg substitution for G1y711 of EF-2 in *Pichia pastoris* did not affect cell growth at normal conditions.

Please replace the paragraph beginning on page 42, line 15 with the amended paragraph that follows below.

To test whether the obtained EF-2 mutants are resistant to DT expression, mutEF2JC307-8, an EF-2 mutant clone (clone 8) of JC307, was transformed with the plasmid DNA of pPIC3-DtA. The construction of pPIC3-DtA was previously described (Woo et al., 2002). Briefly, the DT A chain gene with Bam HI at its 5' end and Not I at 3'was amplified by PCR, inserted into *Pichia pastoris* expression vector pPIC 3 (Invitrogen) and digested with these two enzymes. Integration of pPIC3-DtA allows cytosolic expression of DT chain upon methanol induction. This plasmid DNA had previously been used to transform the GS200 strain of *Pichia pastoris* (Invitrogen) and two of the resulting clones (C3 and C4) were used in the study on tolerance of *Pichia pastoris* to DT (Woo at al., 2002). C3 had been characterized as a non-DT A expressing clone, whereas C4 is a DT A expressing clone. After the transformation with pPIC3-DtA, six mutEF2JC307-8, (mutEF2JC307-8- DtA(1) to (6), clones were randomly picked for analysis of their cytosolic expression of DT A chain and their viability after methanol induction. Cells from single colonies of mutEF2JC307-8-DtA(1) to (6), C3 and C4 were grown in 2m1 YPD (Yeast extracts-Peptone-Dextrose) medium at 30°C overnight before being pelleted down by centrifugation. Cells from each culture were resuspended in YP medium to a density at OD600 nm ± 0.5. Cell suspensions (2 ml) were induced by adding methanol to 1% and incubated at 30°C with vigorous shaking. After methanol induction for 24 hours, cells from 100 µl of each culture were pelleted down and washed with PBS buffer. After this, cells were resuspended in PBS and mixed with protein sample buffer. Finally, the samples were subjected to two cycles of boiling and freezing on dry ice before being analyzed by SDS-PAGE and Western blotting with a DT specific

antibody. The cultures of mutEF2JC307-8-DtA(3) and (5), C3 and C4 were also used for viability assay. This was performed by diluting each culture 104 to 107 fold with PBS buffer, plating 100 µl of aliquot on YPD plate and then counting the colonies appearing on the plates after 3 days incubation at 30°C. The result of SDS-PAGE and Western blotting showed that except for mutEF2JC307-8-DtA(5), all mutEF2JC307-8-DtA clones expressed DT A chain (Fig [[7]]6a). The expression of mutEF2JC307-8- DtA(3) was estimated roughly at 20 µg/ml cell culture. As expected, C3 did not express DT A. Although C4 did express DT A, the protein band was barely visible (Fig [[7]]6b). Before methanol induction, the number of the colony forming units (CFU) per ml of cells was about the same for mutEF2JC307-8- DtA(3) and (5), C3 and C4. After 24 hours methanol induction, the CFU number of mutEF2JC307-8-DtA(3) and (5) and C3 all increased about 103 fold, whereas the CFU number of C4 decreased about 102 (Fig.[[8]]7). This result demonstrated that the expression of DT A chain in the cytosol of cells bearing the mutated EF-2 was not toxic to the cells.

Please replace the paragraph beginning on page 44, line 8 with the amended paragraph that follows below.

In a second attempt to express the bivalent immunotoxin in mutated *Pichia pastoris,* two copies of A-dmDT390-bisFv gene were introduced into mutEF2JC303-5, an EF-2 mutant clone (clone 5) of JC303, which is auxotrophic for histidine and arginine. To build an expression vector with ARG4 selection marker, The AdmDT390- bisFv gene (see Fig. 20) was cloned into the expression vector pBLARGSX3 provided by Professor Cregg and described in Geoffrey et al. (2001). This was done by inserting the final version of A-dinDT390-bisFv gene plus the α-factor signal sequence released from pPICZα (Woo et al., 2002) by Hind III and Not I digestion into pBLARG-SX3 that had been cut with these two restriction enzymes. The resulting construct, pBLARG-A-dmDT390-bisFv (Fig [[9]]8a), together with pPIC9K-AdmDT390-bisFv were electroporated at the same time into mutEF2JC303(5). Transformants expressing these two marker genes were selected on plates containing synthetic complete medium minus arginine and histidine (K.D Medical, Maryland). Eighteen colonies were picked from the selection plate and analyzed for

their expression of the immunotoxin protein. SDS-PAGE showed that they all secreted roughly the same amount of intact immunotoxin protein into induction media. This amount was similar to that secreted from single copy clones: mutEF2JC307-8(2) and JHW#2. As shown in Fig [[10]]9a, three of the selected clones (clones 3, 6, 8) also expressed a smaller, but much more abundant protein that reacted with an anti-DT antibody and had the same size as the monovalent immunotoxin (Liu et al., 2000). The smaller protein is more stable than the intact protein regardless as to whether this protein was produced from a truncated copy of A-dmDT390-bisFv gene or the proteolytically cleaved product of the intact protein. The figure also shows that there were many other smaller proteins in the culture supernatant that reacted with the anti-DT antibody; they were most likely the proteolytic cleaved products of the intact protein. The smallest and also the most abundant one was characterized as the A chain of DT, which is very stable (Collier 1975) and can account for the final product of proteolytic degradation of the intact protein. The degradation also took place inside the cell (Fig. [[10]]9b). Because the A chain is about 1/4 of the size of the intact protein, the amount of the A chain shown on the Western blot indicates that the actual expression level was probably several times higher than the level of intact protein present in the induction medium. A majority of the protein synthesized was probably degraded either before or after secretion out into the medium. Although the double copy clones accumulated the same amount of intact protein in the medium as the single copy clones, the double copy clones produced a larger amount of degraded products, indicating that more gene products had been synthesized. Different measures to control the protein degradation have been employed but the production of the intact protein has not been increased. Thus protein degradation either within or outside the cell is a limiting factor to increase the production of the bivalent immunotoxin.

Please replace the paragraph beginning on page 48, line 15 with the amended paragraph that follows below.

Under these conditions, maximum production of the wild-type expression strain, pJHW #2, is 27.5 mg/L with the total amount of 286.0 mg of the bivalent immunotoxin in 42 hrs of methanol

induction. This level could not be increased beyond 42 hrs of induction. However, under conditions adopted from those for pJHW #2, production the EF-2 mutant strain YYL8-2 continued to increase up to 94 hrs after methanol induction in spite the fact that the initial 10L of culture medium was gradually diluted to 13.4L with methanol and 10% casamino acids solution (see run 5). The total amount of the bivalent immunotoxin of run 5 was 435.5 mg (32 mg/L). This is 1.46-fold greater that the maximum production of pJHW #2. The difference in the production of the bivalent immunotoxin between these two strains is reflected by the methanol consumption rates as shown in Fig. [[11]]10.

Please replace the paragraph beginning on page 51, line 6 with the amended paragraph that follows below.

The construct pPGAPArg-A-dmDT390-bisFv was made by replacing the AOX1 promoter of pBLARG-A-dmDT390-bisFv with P_{GAP} (Fig.[[9]]8b). First, P_{GAP} was amplified from the expression vector pGAPZ A (Invitrogen) by PCR with primers containing sequences of P_{GAP} 5' and 3' ends. The 5' and 3'end primers had a Nhe I and Hind III added respectively. After digestion with Nhe I and Hind III, the PCR products of P_{GAP} were then inserted in pBLARG-A-dmDT390-bisFy that had been cut with these two restriction enzymes to remove the AOX1 promoter. The construct pPGAPHis-A-dmDT390-bisFv (Fig. [[9]]8c) was created by joining DNA fragments from plasmids pPIC9K (Invitrogen) and pPGAPArg-A-dmDT390-bisFv. The plasmid pPIC9K was first cut by Sfu I, after filling in with Klenow Fragment by Not I, then the DNA fragments were separated by agarose gel electrophoresis. The 5.1 kbp fragment containing kanamycin resistant gene, HIS4 gene and 3' AOX1 transcription termination (TT) was isolated and ligated with the plasmid DNA pPGAPArg-AdmDT390-bisFv that had been digested with Not 1 and Sca I to remove the 3'AOXl TT and ARG4 gene.